# Time of day influences immune response to an inactivated vaccine against SARS-CoV-2

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## Methods and materials

### PBMC isolation and flow cytometry

All of the blood samples were collected either at 8AM-10AM or 15PM-17PM. Peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient centrifugation on Ficoll-PaqueTM PLUS (GE Healthcare, USA). For FACS antibody staining, PBMCs were first blocked with Human TruStain FcX™ (Fc Receptor Blocking Solution) (Biolegend, USA) for 15 minutes at room temperature. Following blocking, PBMCs were stained with FITC-CD3 (clone# HIT3a ), AF647-CD4 (clone# RPA-T4), Percp-CD8 (clone# SK1), PE-CY7-CD19 (clone# HIB19), Pacific Blue™-CD56 (clone# HCD56), BV785-CD14 (clone# M5E2), Pecy5.5-CD11b (clone# ICRF44), PE/Dazzle™ 594-CD11c (clone# Bu15), BV510-HLA-DR (clone# L243), BV711-CXCR5 (clone# J252D4), PE-PD-1 (clone# A17188B), BV650-CD45RA (clone# HI100), APC-CY7-CCR7 (clone# G043H7) antibodies for immune phenotyping. For B cell subset classification, PBMCs were stained with PE-CY7-CD19, AF700-CD27 (clone# M-T271), FITC-IgD (clone# IA6-2), APC-CD38 (clone# HIT2), BV421-CD138 (MI15), PE/Dazzle™ 594-CD24 (clone#ML5), APC/Fire™ 750-CD21 (clone#Bu32), BV-605-IgM (MHM-88), AF647-IgG (clone# HIB19), BV785-CD73 (clone#AD2), PE-CY7-CD95 (clone@DX2), BV510-CXCR3 (clone# G025H7), PE-IgA (clone# HM47), Pacific Blue™-CD5 (clone# L17F12) antibodies. All of the FACS antibodies were acquired from Biolegend (USA). The antibodies were diluted at 1:100 and incubated at 4°C for 30 minutes. Cells were then washed with PBS twice. Samples were analyzed by flow cytometry with Cytek™ AURORA. FlowJo (Tree Star, USA)

software was used for FAC data analysis.

**SARS-CoV-2 neutralizing antibodies measurement**

The serum neutralizing antibodies (NAb) for SARS-CoV-2 were detected through chemiluminescent immunoassay using iFlash 2019-nCoV NAb kits (YHLO Biotech Co, Ltd). Briefly, acridinium ester-labeled angiotensin-converting enzyme 2 (ACE2) combined with the receptor binding domain (RBD) of the SARS-CoV-2 spike protein-coated magnetic beads which had bound to SARS-CoV-2 NAb. The complexes were separated under magnetic field. SARS-CoV-2 NAb titers were calculated by an iFlash3000 Chemiluminescence Immunoassay Analyzer (YHLO Biotech Co, Ltd). The titers above 10 AU/mL were considered significant.

**Detection of SARS-CoV-2 specific memory B cells**

To detect SARS-CoV-2 specific B cells, biotinylation of the spike and RBD proteins was performed using biotin-protein ligase reaction kit (Avidity, Bir500A) according to the manufacturer's instruction (1). Biotinylated protein antigens were then individually multimerized with fluorescence labeled streptavidin. Brifely, recombinant SARS-CoV-2 spike protein (R&D) was mixed with BV510-streptavidin (BioLegend) at 10:1 ratio and BV785-streptavidin (BioLegend) at 18:1 ratio at 4°C for 1 hour. Recombinant SARS-CoV-2 RBD protein (R&D) was mixed with BV421-streptavidin (BioLegend) at 20:1 ratio at 4°C for 1 hour. The antigen probes prepared individually above were then mixed in 50mM free d-biotin (Macklin) in PBS to ensure minimal cross-reactivity. PE-streptavidin (BioLegend) was used as a decoy probe to gate out

SARS-CoV-2 non-specific streptavidin-binding B cells. PBMC ($1\times10^7$) from 63 volunteers were prepared in 5ml-round bottom polystyrene tubes in RPMI 1640 medium supplemented with 10% of fetal bovine serum. After a rest of 2h at 37°C and 5% CO2, cells were stained with Zombie Red (BioLegend) in PBS at 4°C for 20min ans washed. Cells were then stained with 50 µl of antigen probe cocktail containing 100 ng of spike, 50 ng of RBD and 20 ng of streptavidin-PE at 4°C for 30 min. Cells were washed with PBS and then stained with the following antibody cocktail: CD3-Pacific Blue™ (clone# HIT3a), CD19-PE-CY7 (clone# HIB19) , CD27-AF700 (clone# M-T271), IgD-FITC (clone# IA6-2), CD38-APC (clone# HIT2), all from BioLegend at 1:100 dilution. Samples were aquired on Cytek™ AURORA and analyzed using FlowJo 10 (Tree Star).

**Cytokine measurement**

For cytokines detection, 25 µL plasma was incubated with 25 µL PE detection reagent for 2.5 h according to the manufacture's instruction (Saiji Biotechnology Co. Ltd). The cytokine measurement was performed using a Beckman Coulter Multidetector (Beckman Coulter, Fullerton).

**Statistical analysis**

Statistical analysis was performed using Prism 5.0. Comparisons were assessed using the Mann-Whitney U test, Wilcoxon rank sum test, Student's t-test, paired Student's t-test or One-way ANOVA followed by Bonferroni's multiple comparison

post-test as appropriate. $P$ values $< 0.05$ were considered as statistically significant.

Supplementary data

**Table S1. Demographics and adverse events of morning and afternoon vaccination groups**

| | | Morning group | Afternoon group | Total |
|---|---|---|---|---|
| Participants, N | | 33 | 30 | 63 |
| Age, median [IQR] | | 26 [24, 27] | 26 [25, 28] | 26 [24, 28] |
| Gender (male %) | | 13 (39.4) | 13 (43.3) | 26 (41.3) |
| Participants with adverse events, N (%) | | 8 (24.2%) | 7 (23.3%) | 15 (23.8%) |
| Injection site symptoms | Induration, N (%) | 2 (6.1%) | 1 (3.3%) | 3 (4.8%) |
| | Swollen, N (%) | 2 (6.1%) | 1 (3.3%) | 3 (4.8%) |
| Systematic symptoms | Fever, N (%) | 1 (3.0%) | 1 (3.3%) | 2 (3.2%) |
| | Fatigue, N (%) | 0 (0.0%) | 2 (6.7%) | 2 (3.2%) |
| | Headache, N (%) | 1 (3.0%) | 0 (0.0%) | 1 (1.6%) |
| | Myalgia, N (%) | 0 (0.0%) | 1 (3.3%) | 1 (1.6%) |
| Digestive symptoms | Diarrhea, N (%) | 1 (3.0%) | 1 (3.3%) | 2 (3.2%) |
| | Vomiting, N (%) | 1 (3.0%) | 1 (3.3%) | 2 (3.2%) |
| | Nausea, N (%) | 0 (0.0%) | 1 (3.3%) | 1 (1.6%) |
| Respiratory symptoms | Runny nose, N (%) | 2 (6.1%) | 2 (6.7%) | 4 (6.3%) |
| | Cough, N (%) | 1 (3.0%) | 1 (3.3%) | 2 (3.2%) |
| | Stuffy nose, N (%) | 0 (0.0%) | 1 (3.3%) | 1 (1.6%) |
| | Sore throat, N (%) | 1 (3.0%) | 1 (3.3%) | 2 (3.2%) |

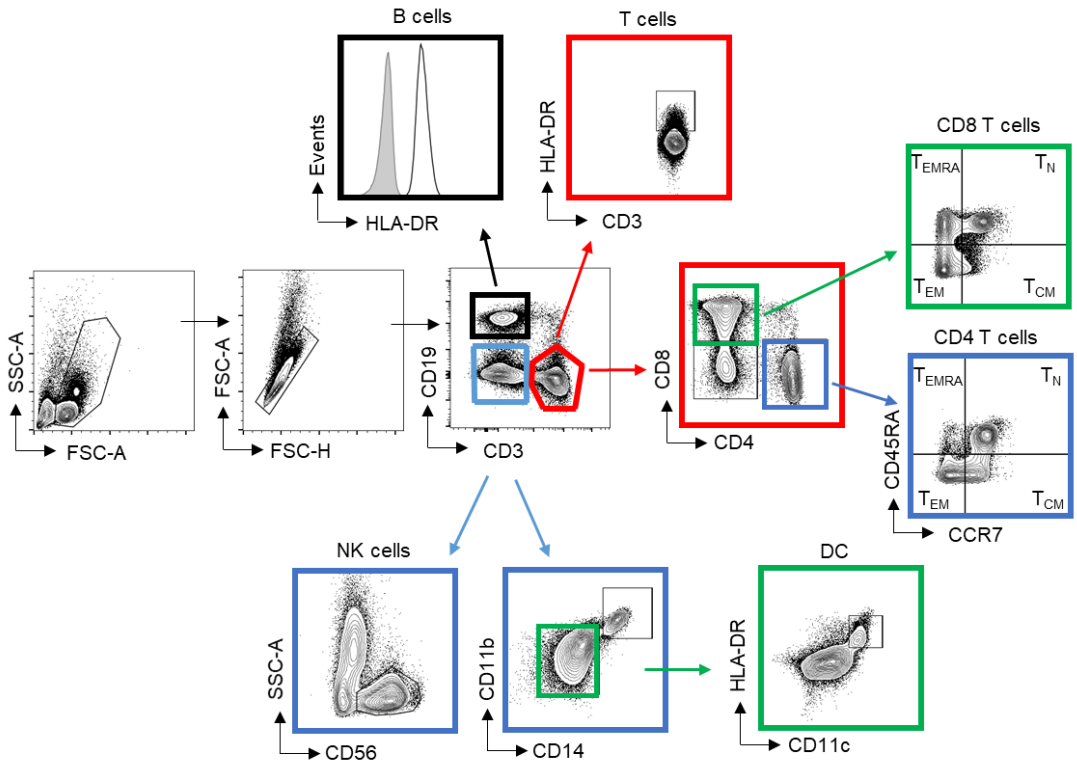


**Fig. S1. Gating strategy.** For immune phenotyping, PBMCs isolated from the participants in this study were stained with antibodies as described in the method section. The immune cell populations are defined as: T cells: $CD3^{+}CD19^{-}$, B cells: $CD3^{-}CD19^{+}$, CD4 T cells: $CD3^{+}CD4^{+}$, CD8 T cells: $CD3^{+}CD8^{+}$, double negative (DN) T cells: $CD3^{+}CD4^{-}CD8^{-}$. NK cells: $CD3^{-}CD19^{-}CD56^{+}$, monocytes: $CD3^{-}CD19^{-}C14^{+}CD11b^{+}$, Dendritic cells: $CD3^{-}CD19^{-}CD14^{-}CD11b^{-}CD11c^{+}HLA\text{-}DR^{+}$, follicular helper T cells (Tfh): $CD3^{+}CD4^{+}PD\text{-}1^{+}CXCR5^{+}$. For subsets of T cells: native T cells ($T_N$): $CD45RA^{+}CCR7^{+}$, effector memory T cells ($T_{EM}$): $CD45RA^{-}CCR7^{-}$, central memory T cells ($T_{CM}$): $CD45RA^{-}CCR7^{+}$, terminally differentiated T cells ($T_{ERMA}$): $CD45RA^{+}CCR7^{-}$.

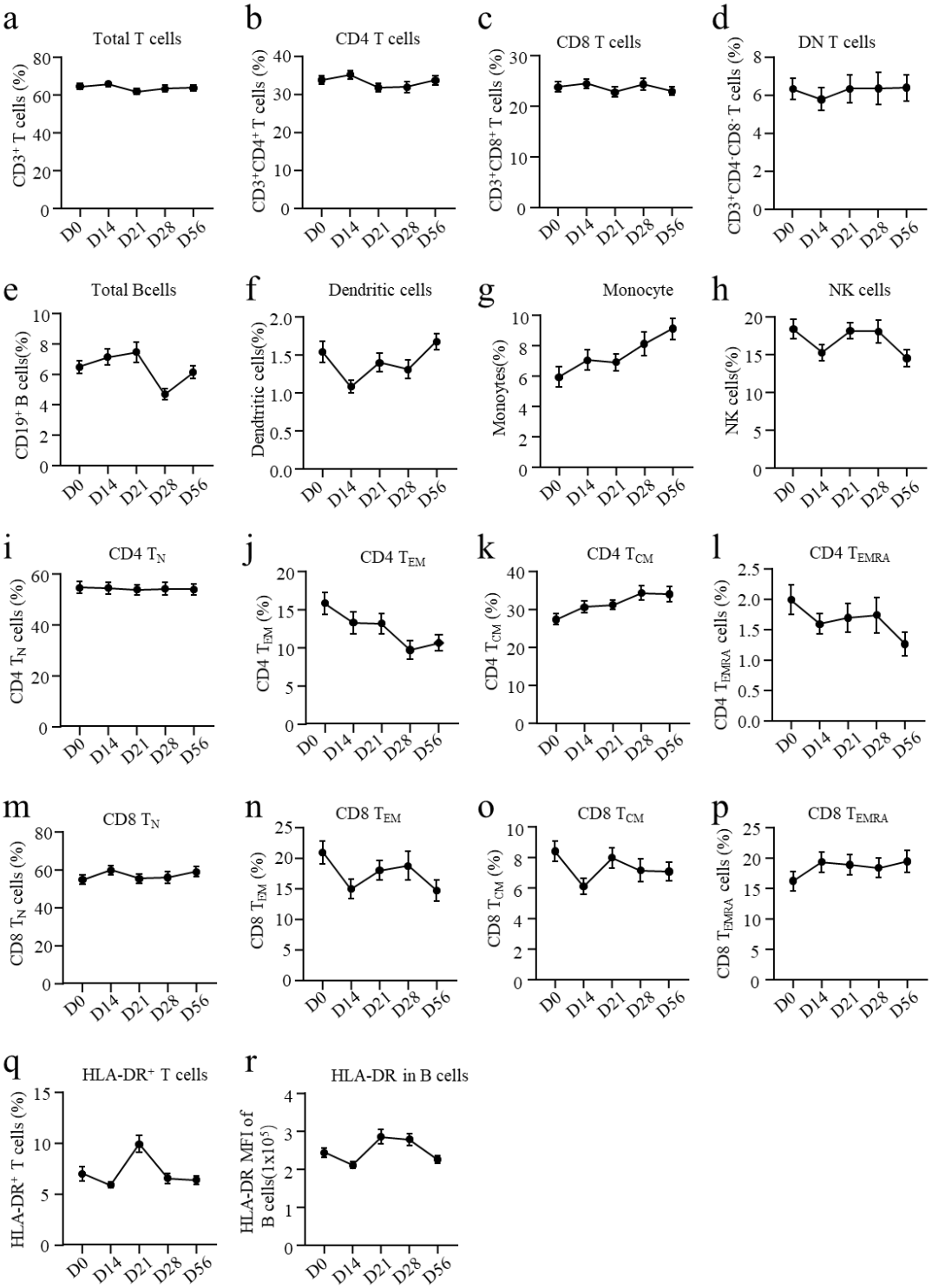


**Fig. S2. Immune cell response to BBIBP-CorV inactivated vaccine**. All the participants were vaccinated with first dose of BBIBP-CorV vaccine on day (D) 0 and second dose on D28. Blood samples were collected on D0, D14, D21, D28, D56. Dynamic changes of immune cell percentages were measured by flow cytometry. The

gating strategy is shown as in Fig. S1. DN: double negative T cells. $T_N$: naïve T cells, $T_{EM}$: effector memory T cells, $T_{CM}$: central memory T cells, $T_{EMRA}$: terminally differentiated T cells. MFI: mean fluorescence intensity.

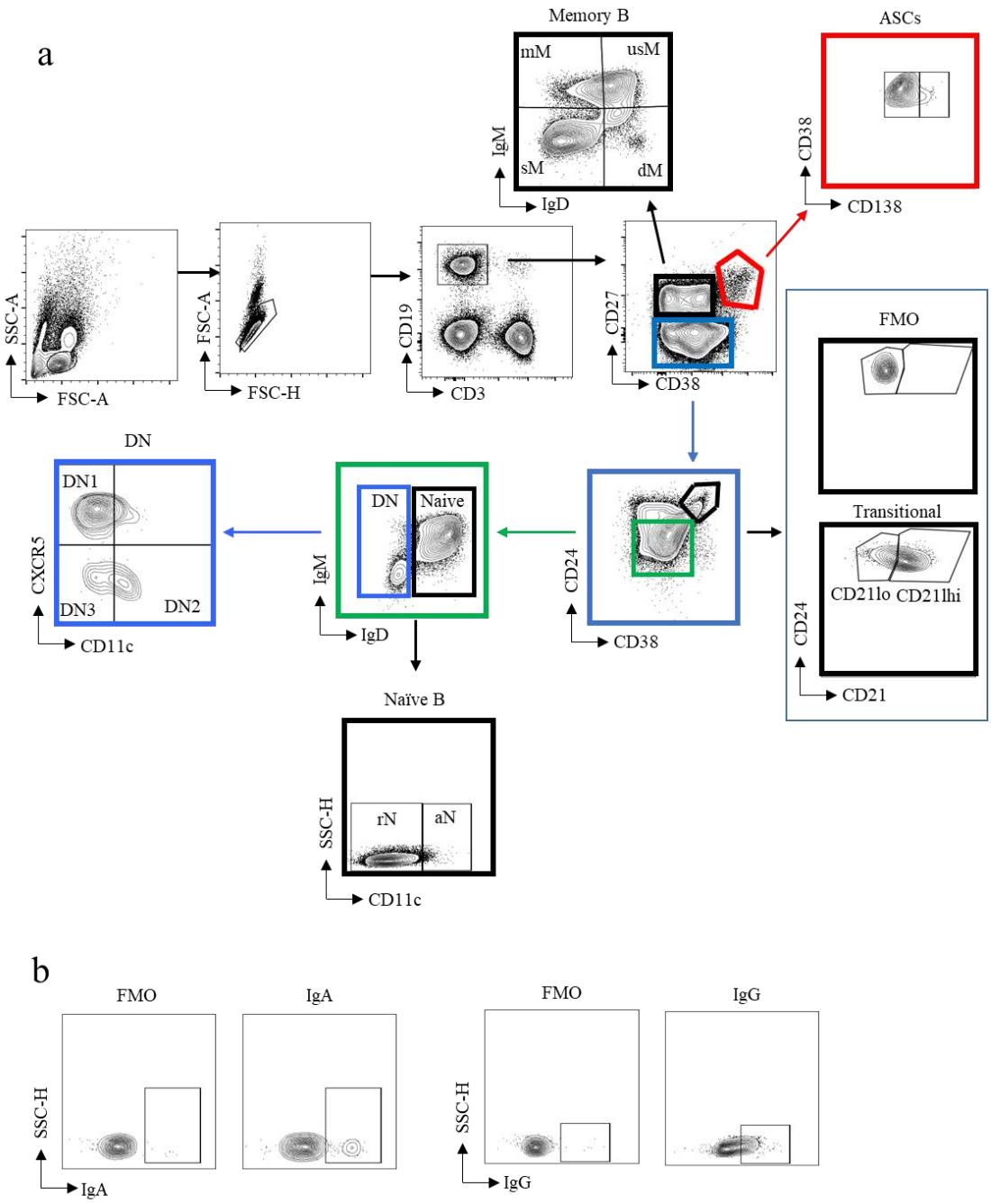


**Fig. S3. Gating strategy of B cell subsets.** PBMCs isolated from the participants were stained with FACS antibodies as described in the method section. For B cell subset classification, cells were first gated on $CD3^{-}CD19^{+}$ B cells. B cell subsets were defined as: antibody secreting cells (ASC): $CD27^{+}CD38^{++}$, naïve B cells: $CD27^{-}CD38^{-}IgD^{+}$, active naïve B cells (aN): $CD27^{-}CD38^{-}IgD^{+}CD11c^{+}$), memory B cells: $CD27^{+}CD38^{low/-}$

(memory B cell subsets: switched memory B cells (SM): $CD27^{+}CD38^{-/low}IgM^{-}IgD^{-}$, $IgM^{+}$ memory B cells: $IgM^{+}IgD^{-}$, $IgD^{+}$ memory B cells: $IgM^{-}IgD^{+}$, unswitched memory B cells (UN-SM): $IgM^{+}IgD^{+}$), double negative (DN) B cells: $CD27^{-}IgD^{-}CD38^{+}$, DN B cells were further separated into DN1, DN2 and DN3 based on the expression of CD11c and CXCR5. DN1: $CD11c^{-}CXCR5^{+}$, DN2: $CD11c^{-}CXCR5^{+}$, DN3: $CD11c^{-}CXCR5^{+}$. Transitional B cells (Tr): $CD27^{-}CD38^{int}CD24^{+}$.

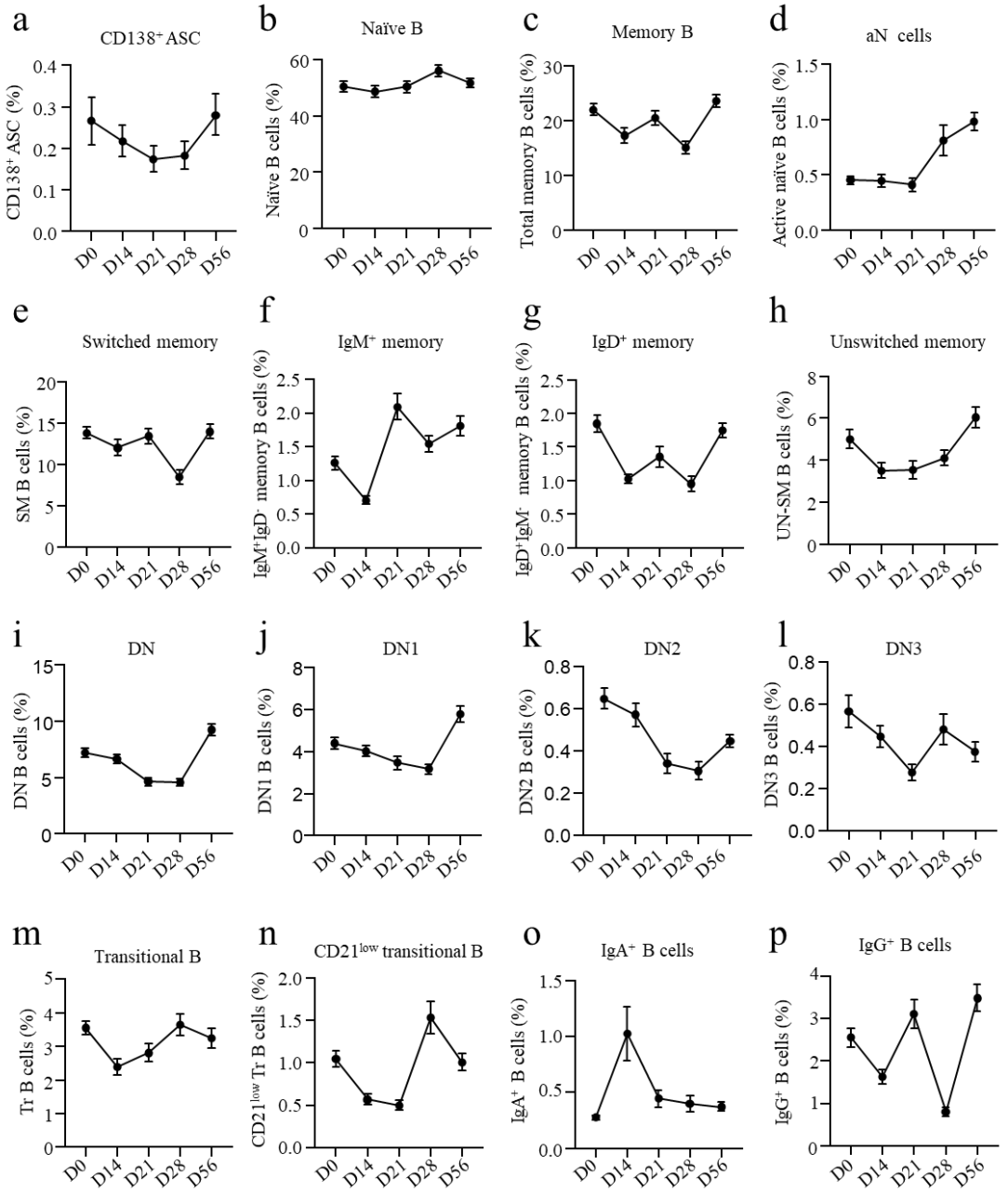


**Fig. S4. B cell response to BBIBP-CorV inactivated vaccine**. All the participants were vaccinated with first dose of BBIBP-CorV vaccine on day (D) 0 and second dose on D28. Blood samples were collected on day (D) 0, D14, D21, D28, D56. B cell subpopulations were measured and quantified by flow cytometry. The gating strategy was shown as in Fig. S3.

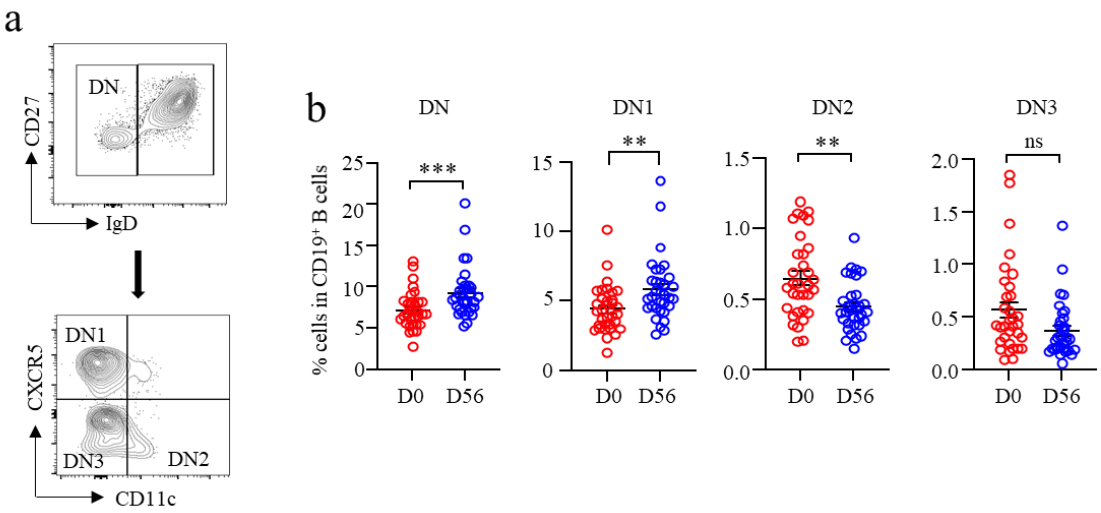


**Fig. S5. Extrofollicular B cell response to the inactivated vaccine.** The participants were vaccinated by BBIBP-CorV inactivated vaccine on day(D) 0 and day 28. Blood samples from day (D) 0 and D56 were analyzed by flow cytometry. Cells were first gated $CD3^{-}CD19^{+}$ population and then further gated on $CD27^{-}IgD^{-}$ double negative (DN) B cells. DN B cells were further characterized based on the expression of CD11c and CXCR5 as defined in Fig. S3. ns: not significant. Data are mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$ by Student's t-test.

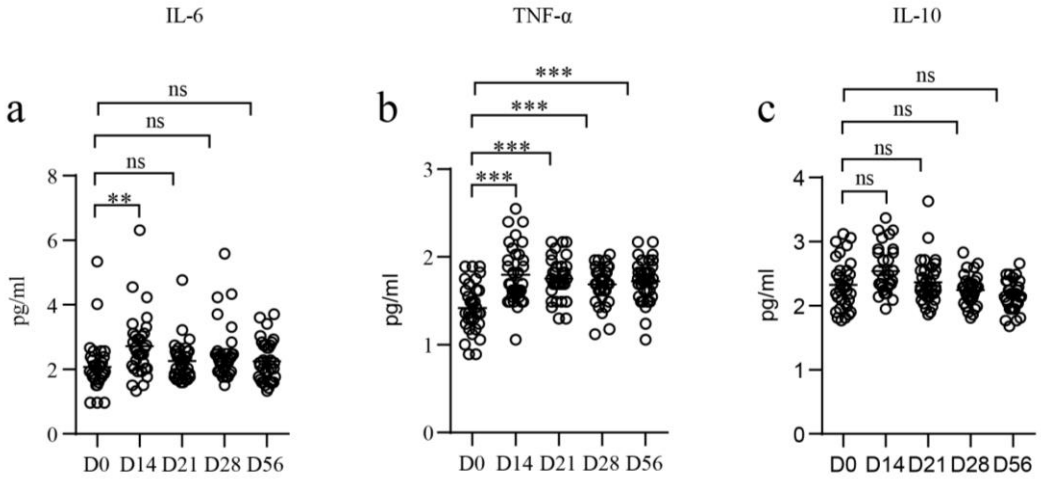


**Fig. S6. Cytokine response to BBIBP-CorV inactivated vaccine**. Health care workers were vaccinated by the inactivated vaccine of BBIBP-CorV on day(D) 0 and day 28. Serum samples were collected from participants on day D0, D14, D21, D28, D56 and cytokines of IL-6, TNF-α and IL-10 were measured. ns: not significant. Data are mean ± SEM. ***P*<0.01, ****P*<0.001 by One-way ANOVA.

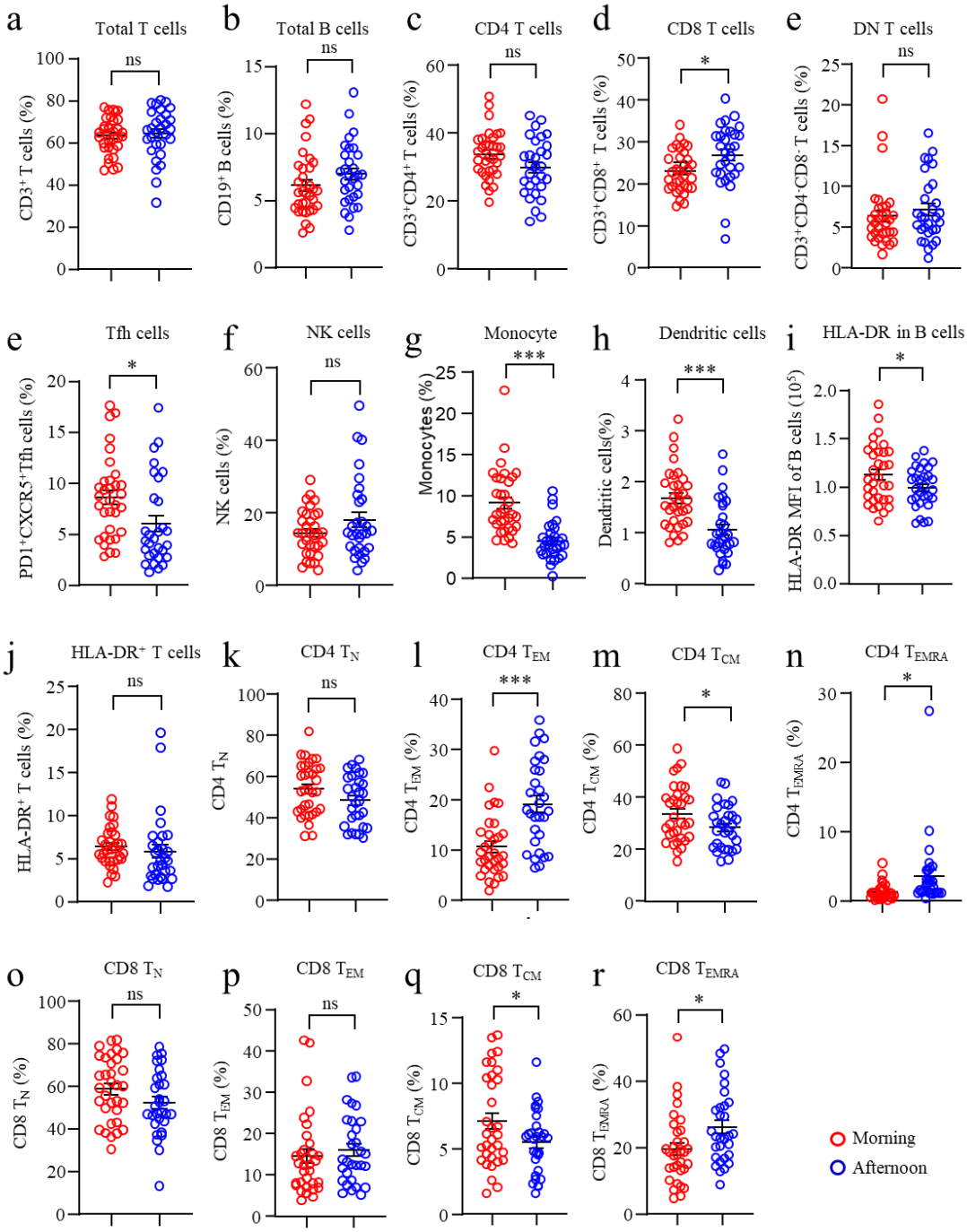


**Fig. S7. Time of day influences immune response to the BBIBP-CorV inactivated vaccine**. Blood samples were collected from morning vaccination group (8AM-10AM) or afternoon vaccination group (15PM-17PM) on day 56. PBMCs were isolated and stained with FACS antibodies as described in the method section. Data were acquired by flow cytometry. DN: double negative T cells. $T_N$: naïve T cells, $T_{EM}$: effector

memory T cells, $T_{CM}$: central memory T cells, $T_{EMRA}$: terminally differentiated T cells. MFI: mean fluorescence intensity. ns: not significant. Data are mean ± SEM. *$P$<0.05, ***$P$<0.001 by Student's t-test.

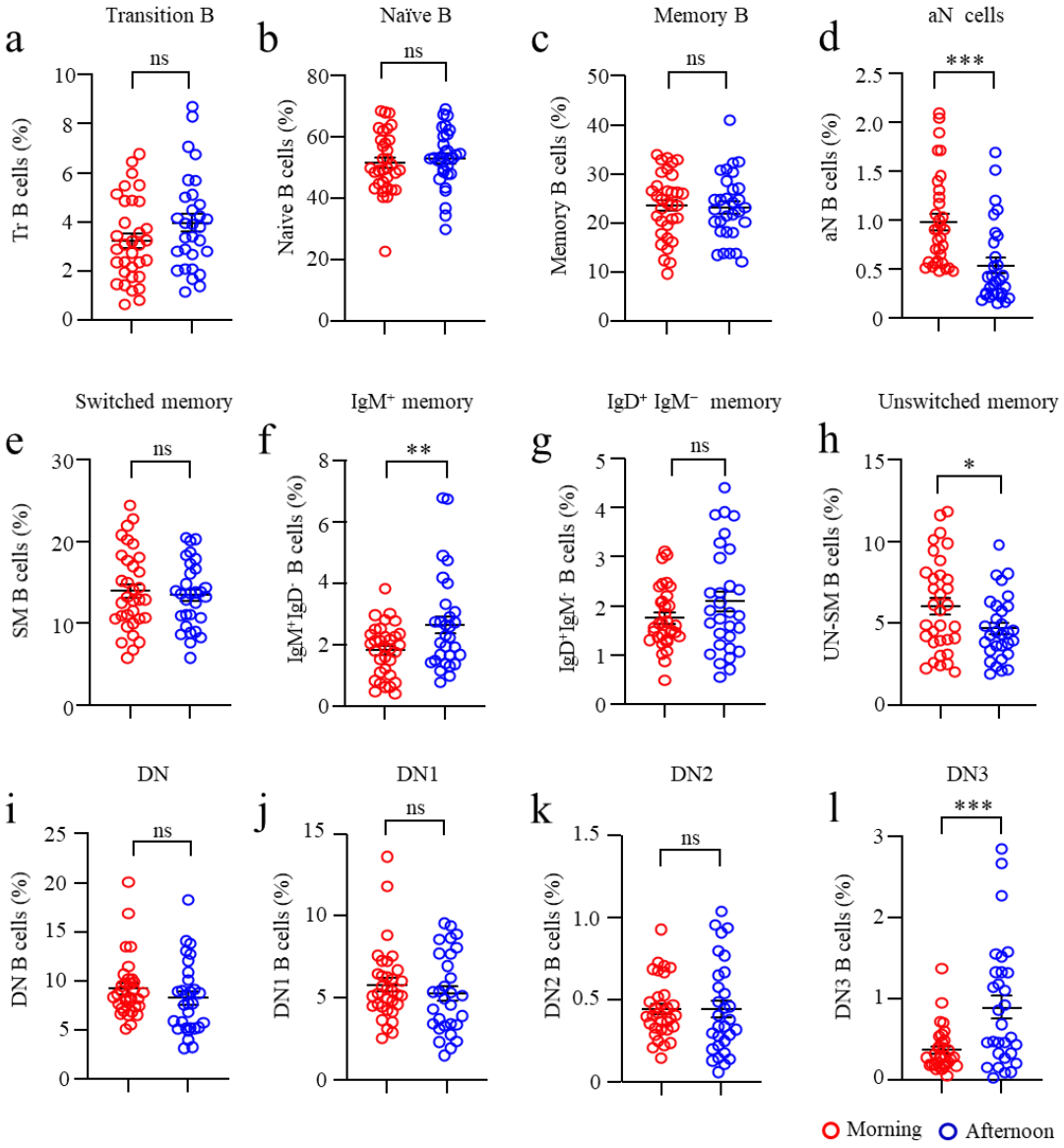


**Fig. S8. Time of day influences B cell response to the BBIBP-CorV inactivated vaccine.** Blood samples collected from morning vaccination group (8AM-10AM) or afternoon vaccination group (15PM-17PM) on day 56 were processed for PBMCs isolation. Cells were then stained with FACS antibodies as described in the method section. Data were acquired by flow cytometry. Gating strategy was performed as in Fig. S3. ns: not significant. Data are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student's t-test.

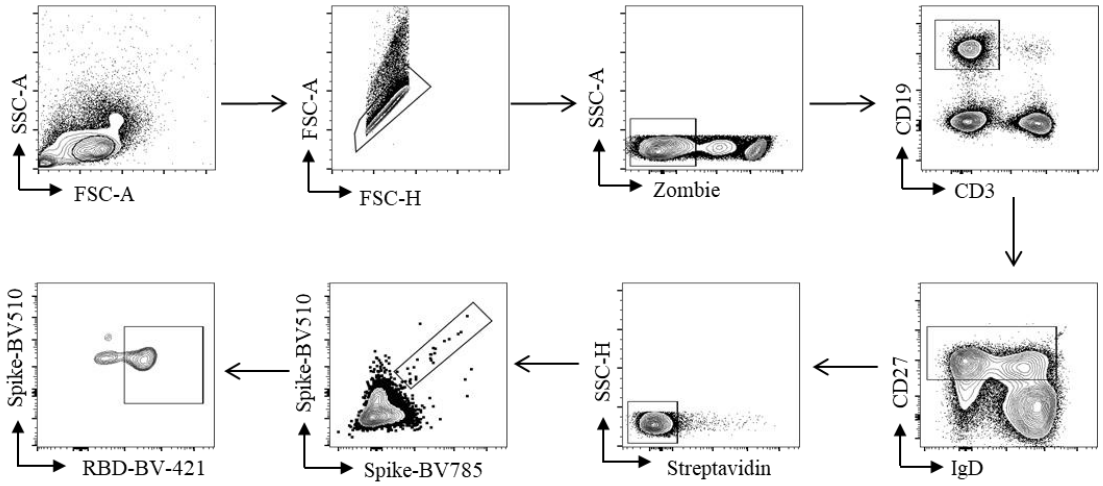


**Fig. S9. Flow cytometry identification and gating strategy for SARS-CoV-2 specific memory B cells**. Participants were vaccinated on day 0 and day 28. PBMCs were collected on day 56. SARS-CoV-2 specific memory B cells were identified by flow cytometry. Zombie was used to gate out dead cells. Cells were then gated on $CD3^-$ $CD19^+$ B cells and further gated on $CD27^+$ memory B cells. Unspecific binding of streptavidin was excluded.

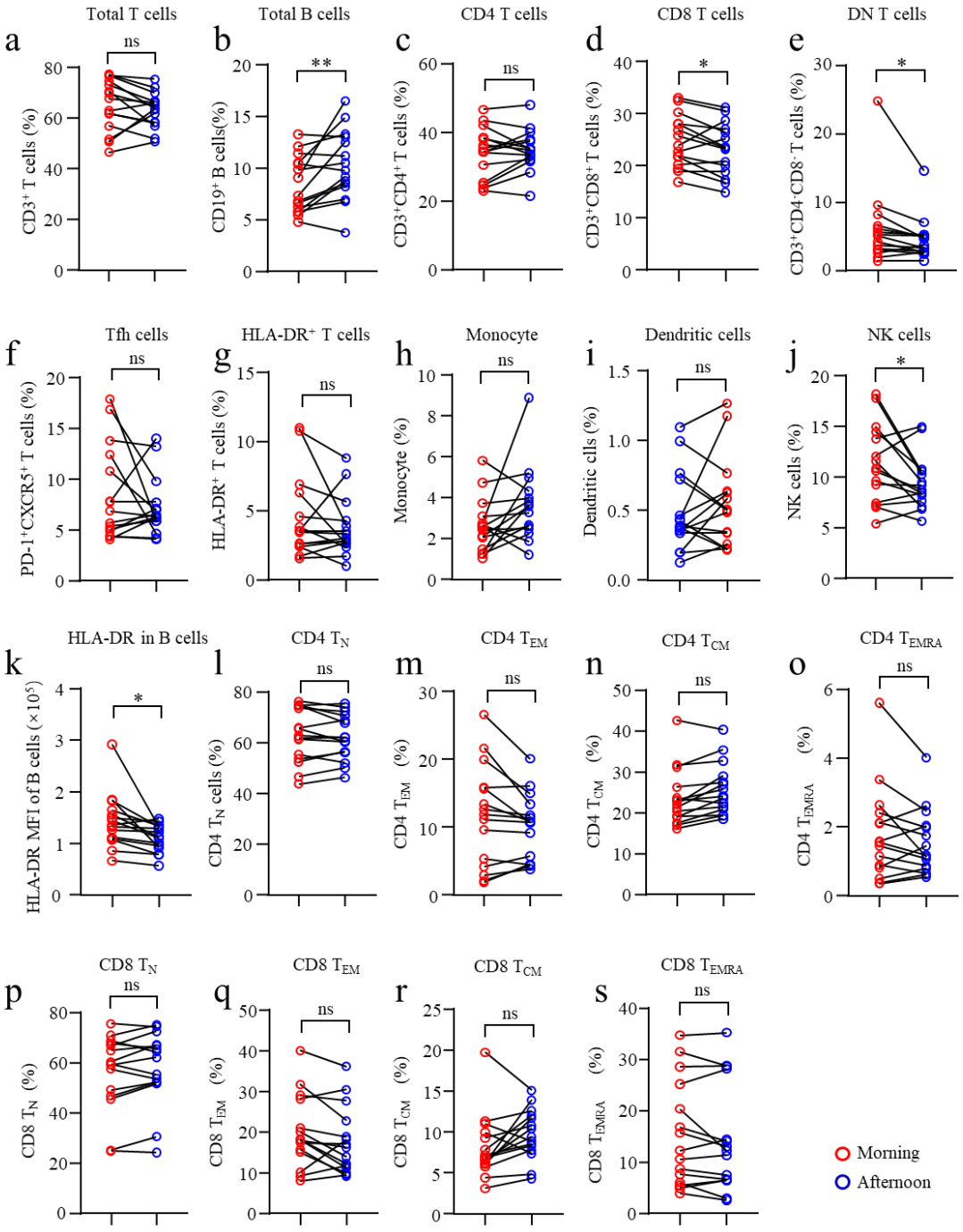


**Fig. S10. Dynamics of immune cells in the blood from morning and afternoon**. Blood samples were collected from the 15 volunteers (Male/female=4/11, age=28.6± 6.17) twice in a same day: morning time at 8AM-10AM, afternoon time at 15PM-17PM. PBMCs were isolated and stained with FACS antibodies as in the method section. Data were acquired by flow cytometry. Gated strategy was performed as in Fig. S1. DN:

double negative T cells. $T_N$: naïve T cells, $T_{EM}$: effector memory T cells, $T_{CM}$: central memory T cells, $T_{EMRA}$: terminally differentiated T cells. ns: not significant. *$P$<0.05, **$P$<0.01 by paired Student's t-test.

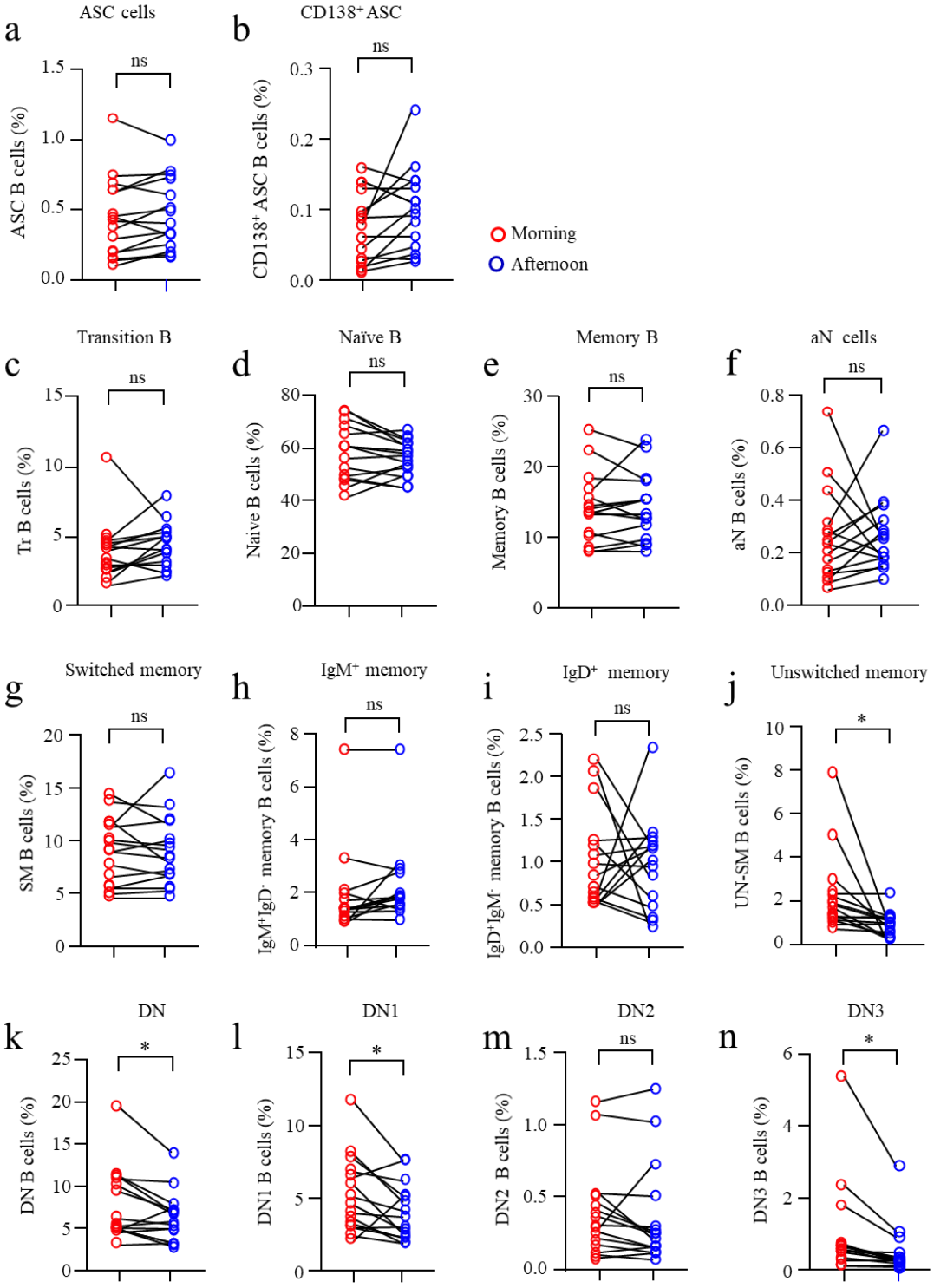


**Fig. S11. Dynamics of B cell subsets in the blood from morning and afternoon.** Blood samples were collected, processed and measured as in Fig. S10. aN: active naïve B cells, DN: double negative, SM: switched memory B cells, UN-SM: unswitched B cells, Tr: transitional B cells, ns: not significant. *$P$<0.05 by paired Student's t-test.